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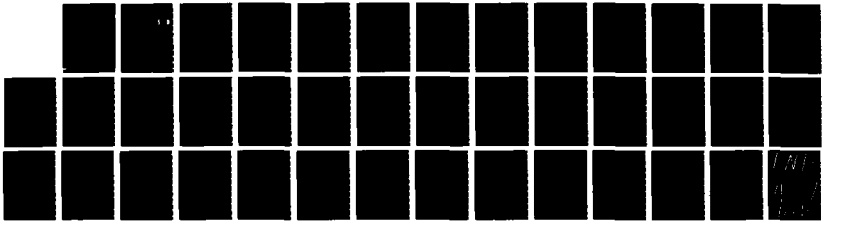

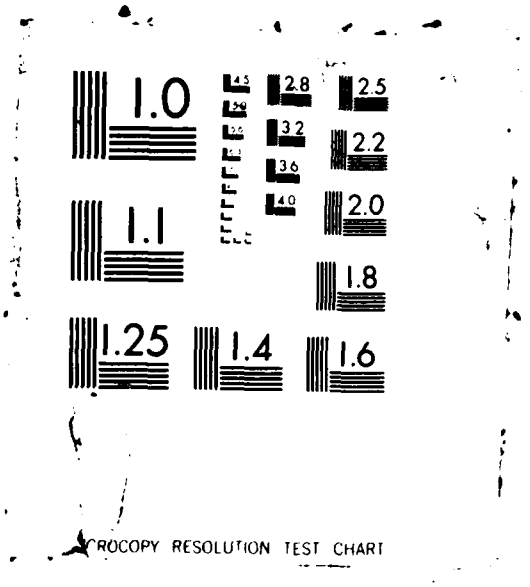
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STUDIES ON TYPHUS AND SPOTTED FEVER

FINAL REPORT

by

Charles L. Wisseman, Jr., M. D.

September 1986
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This multifaceted research program was a planned coordinated program in which the individual components were designed to converge ultimately on the militarily significant mission of improved methods for the prevention, control and treatment of rickettsial diseases. Studies were concentrated primarily on typhus group rickettsiae, but some observations were made on spotted fever and scrub typhus group organisms and on Rochalimaea quintana as well. These included, but were not limited to, the development of modern quantitative

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methodology and studies on the biology of the infection cycle, basis for immunity in typhus so that immunological requirements of vaccines could be defined, analysis of surface components of typhus organisms that might be involved in protective immunity, elementary genetics and chemotherapy.

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SUMMARY

This multifaceted research program was a planned coordinated program in which the individual components were designed to converge ultimately on the militarily significant mission of improved methods for the prevention, control and treatment of rickettsial diseases. Studies were concentrated primarily on typhus group rickettsiae, but some observations were made on spotted fever and scrub typhus group organisms and on <u>Rochalimaea quintana</u> as well. These included, but were not limited to, the development of modern quantitative methodology and studies on the biology of the infection cycle, basis for immunity in typhus so that immunological requirements of vaccines could be defined, analysis of surface components of typhus organisms that might be involved in protective immunity, elementary genetics and chemotherapy.

CONTENTS

I. OVERVIEW.

This multifaceted research program was a planned coordinated program in which the individual components were designed to converge ultimately on the militarily significant mission of improved methods for the prevention, control and treatment of rickettsial diseases. Studies were concentrated primarily on typhus group rickettsiae, but some observations were made on spotted fever and scrub typhus group organisms and on Rochalimaea quintana as well.

Because of the relatively unsophisticated state of rickettsial methodology and of basic knowledge of rickettsial biology, immunity, genetics, etc. at the time this contract was initiated, several lines of investigation relevant to attaining the primary mission were followed. These included, but were not limited to, the following major categories.

A. Methodology: the development of highly reproducible, predictable methods for quantitation of rickettsiae, for infecting cells, for measuring rickettsial growth and studying the infection cycle and for observing and measuring the effects of various factors, antimicrobials, cells and conditions on rickettsial growth.

B. Biology of the infection cycle.

C. Basis for immunity in typhus so that the immunological requirements of vaccines could be defined.

D. Analysis of surface components of typhus organisms that might be involved in protective immunity.

E. Examination of the rickettsial genome, degree of relatedness among typhus and spotted fever group rickettsiae based on DNA:DNA hybridization, and elementary observations on classical genetics.

F. Chemotherapy of rickettsial diseases: search for rickettsiacidal antimicrobials.

Numerous miscellaneous observations were made in the course of these studies.

As documented in the sections which follow, considerable progress was made in most of these areas. The goal of defining

the immunological objectives or requirements of a typhus vaccine, in terms of antibody and cell mediated immunity and of rickettsial surface immunogens, appeared to be within practical operational reach. Unfortunately, this contract was terminated at this crucial point, with work along these lines being continued as a major military in-house research effort (NAMRI).

II. SPECIFIC REPORTS

A. Methodology. Rickettsiological research was badly lagging behind other branches of microbiology because of the lack of adequate modern methods. Early in this contract a major effort was made to develop or adapt methods which would permit easy accurate quantitation of organisms, purification of rickettsial suspensions, production of suspensions enriched in viable organisms, selection of clones, and production of reproducible, defined and predictable infections, among others. This was successful in many ways and a totally new set of laboratory procedures became the standard operating system for the laboratory. The new methodology permitted studies which were previously impossible or extremely laborious. The use of these methods or their adaptation to special problems made possible much of the research described in the sections below. Many visitors to the laboratory were trained in the new methodology. Some of the important developments and adaptations are briefly listed below.

1. **Quantitation of Rickettsiae.** Methods were developed for counting the absolute number of rickettsial bodies (RLB), first in purified suspensions and later in suspensions containing some host cell debris. The production of plaques by rickettsiae in host cell monolayers under agarose, first described by Kodova, confirmed by others and developed for easy routine use by Wike et al, was adapted for the routine titration of rickettsiae in seeds, purified suspensions and animal tissues. Simultaneously with others, it was found that a close estimate of the absolute number of viable rickettsiae in a suspension could be obtained by centrifuging the plaque plates under standardized conditions at the time of inoculation (cPFU). The RLB/cPFU ratio gave an estimate of the relative proportions of dead and viable rickettsiae in a preparation - a very valuable descriptor of a preparation. The interference of tissue debris, presumably largely cell membranes, with plaque titrations was recognized and methods were developed to minimize this interference.

2. **Pure Culture Principle in Rickettsiology: Plaque Purification and Cloning of Rickettsiae and the Elimination of Extraneous Agents from Rickettsial Seeds.** In previous years, rickettsiae were propagated in the yolk sac of conventional embryonated chicken eggs, monitored for microbial sterility by a routine set of cultures. All rickettsial seeds were prepared in this manner. When the plaque methods, using monolayers of chicken embryo cells obtained from conventional eggs which had passed routine sterility checks, were being adapted to this laboratory, "plaques" were frequently noted in uninoculated plates. This led to an investigation which revealed that all rickettsial seeds on hand, passed and prepared in conventional

eggs were contaminated, sometimes very heavily, with extraneous agents, some of which were bacteria that did not grow in the various media used for sterility checks. Some of these bacteria were very small, stained red with Gimenez stain and closely resembled rickettsiae on smear. This meant that much work on rickettsiae in the past had been done with preparations containing unsuspected non-rickettsial agents.

A major effort was made to rectify this and to introduce the "pure culture" principle into rickettsiology. Specific pathogen-free eggs were then becoming available commercially on a routine basis. These were used to prepare tissue cultures, plaquing plates and rickettsial seeds. Methods were developed for isolating rickettsiae from a single plaque and to pass it through a series of plaque purification steps. This was done for every rickettsial strain of the typhus and spotted fever groups in the laboratory - an extremely laborious procedure. It has become routine with new strains introduced into the laboratory and for isolates from field materials.

The same plaque picking techniques were subsequently used to clone strains with various properties, e.g., antibiotic resistance.

All of the work described in this report was performed with plaque purified strains.

3. **Purification of Rickettsiae and Enrichment of Suspensions for Viability.** A simple 3 stage method for the purification of viable or formalin-killed rickettsiae from large or small quantities of yolk sac suspension was developed, as follows.

a. The initial "sucrose batch" step eliminated much lipid and solid material and reduced the bulk of the centrifuged pellet by about 90%. It consisted of mixing the yolk sac suspension with a predetermined amount of 50% sucrose solution and centrifuging. Large quantities of yolk sac suspension could be reduced to manageable volumes in a single step.

b. The second step was aggregation of yolk sac particles from the resuspended pellet from "a" with a mixture bovine serum albumin and rabbit anti-yolk sac antiserum, followed by removal of the aggregates by low speed centrifugation. Rickettsiae in the supernatant were pelleted by centrifugation and resuspended in a reduced volume.

c. The final step was centrifugation through a discontinuous Renografin gradient, after the method of Dasch et al, one or more times. It was found that formalin-killed rickettsiae yielded a single rickettsial band whereas "viable" suspensions yielded two bands: (1) a "heavy" band consisting largely of non-

viable organisms and (2) a light band greatly enriched for infectious organisms (RLB/cPFU ratios of <2:1).

This flexible method, adaptable to many purposes, became a vital addition to the routine methodology of the laboratory.

4. Methods for the Production of Reproducible, Predictable Infections of Cells and for Studying Infected Cells. Two methods for infecting cells with rickettsiae in a defined and predictable way were developed and characterized in detail. Both methods depended upon having available multiple aliquots of highly characterized rickettsial seeds.

a. Infection in suspension. Host cells settling at 1 x g through a suspension of rickettsiae under standard conditions of time, temperature and medium composition collide with rickettsiae in a predictable statistical way, depending upon the concentration of rickettsiae. Some of these collisions will result in attachment of the rickettsiae and subsequent infection of the cell. Under standardized conditions, it was possible reproducibly to produce any desired predetermined degree of infection with a high degree of accuracy. At low percentages of infected cells, the distribution of rickettsiae within cells followed the Poisson distribution rather closely and could be calculated simply from the percent cells infected. A relationship was found to exist between the percent cells infected and the plaque titer of the rickettsial suspension, permitting the establishment of a standard curve which relates the two. Thus, a very rapid estimate of the titer of a rickettsial suspension could be obtained within an hour. This method formed the foundation for producing infected cells for many types of study.

b. Infection of monolayers of cells. In this system, as in the case of host cells attached to the substrate in slide culture chambers or plates, rickettsiae settle upon the attached host cell to produce infection. The kinetics are somewhat different from those described above, but quantitative relationships and conditions were worked out for the production of predictable reproducible infections. This method was especially useful for producing infection in macrophages which are difficult to obtain in suspension.

Adaptation of the slide culture chambers (Lab-Tek) to the study of infected cells by light microscopy was an important technical development for the study of rickettsial growth cycles (see below) and the influence of various conditions and factors on rickettsial growth.

B. Characteristics of Rickettsial Infection Cycles, with Ultrastructural Obser ations. A systematic quantitative study of the infection cycle of typhus and spotted fever group rickettsiae was made by light microscopy of infected cells, mostly in CE

cells, in slide chamber cultures and by electron microscopy of infected cells from cultures in plastic petri dishes, monitored by light microscopy of parallel slide chamber cultures.

R. prowazekii was found to undergo one type of infection cycle in the cytoplasm of infected cells in which the classical stages of bacteria growing in fluid medium could be identified: a variable lag phase depending upon the inoculum, a phase of logarithmic growth and a stationary phase with accompanying morphological changes as might be expected from bacterial cultures. The organisms remained within the host cell, which showed very few pathological ultrastructural changes until very late in the infection, until the cytoplasm was felled with organisms, whereupon the host cell membrane broke down an released the contained organisms into the medium from which they could initiate infection in other cells. At 32 C, the cycle from initial infection to final release required about 48 h. The division time in the log phase was about 9-10 hours. Under standardized conditions, the infection cycle was extremely predictable and cultures in any predetermined desired stage of growth could be obtained at will - a powerful tool for many kinds of study. All strains of R. prowazekii tested exhibited this type of infection cycle. These included the old established Breinl strain, a freshly isolated Burundi strain and the flying squirrel strains.

Rickettsia mooseri (established and recent isolates) and all established species of the spotted fever group underwent a different type of infection cycle - a spreading type infection. Instead of remaining within the host cell until it finally lost plasma membrane integrity, these organisms began to escape from their host cells without causing apparent damage very soon after infection was established and spread to uninfected cells in the culture, causing a progressive increase in percent cells infected. Within the constraints imposed by the constantly spreading infection, it was possible to identify roughly comparable growth stages. Late in the infection cycle of R. rickettsii ultrastructural studies showed that the endoplasmic reticulum began to swell into large cisternae trapping many organisms and changing the character of the infection.

As the studies on infection cycle were proceeding, ultrastructural studies were carried out on members of the genus Rickettsia. These showed the members of the typhus and spotted fever groups possess cell envelopes comparable in structure t o those of certain gram negative bacilli whereas R. tsutsugamushi exhibited a marked thickening of one of the inner layers of the,outer membrane. An important observation was that under appropriate conditions, an electronlucent "slime layer" of substantial proportions could be demonstrated to surround the rickettsiae.

C. Basis of Immunity in Typhus and Formulation of Immunological Objectives of Vaccines.

1. Conceptual Background Derived from Work in Previous Contract (skeletal summary of major themes): Prior to the development of modern knowledge about the phenomenon of cell mediated immunity in general, immunological studies of typhus infections concentrated on antibodies and antibody responses to typhus vaccines were assumed to be a correlate of vaccine potency. Much emphasis was placed on toxin neutralizing antibodies as a correlate of protective immunity. Under a previous contract we studied the opsonizing activity of typhus convalescent serum for enhanced phagocytosis by polymorphonuclear leukocytes and human peripheral blood derived macrophages. One of the striking findings was that both virulent R. prowazekii and R. mooseri (= R. typhi), upon entry into macrophages in culture were not destroyed but, instead, multiplied and destroyed the macrophage. However, if these rickettsiae were first treated with typhus convalescent serum, they were more avidly phagocytized and were destroyed rapidly by the macrophages which survived the encounter - the first demonstration of an antibody-mediated mechanism which might be important in controlling typhus infections according to existing concepts. Opsonizing antibodies (for PMNs) were found to develop in the serum from patients with louse-borne typhus fever, subjects inoculated with the live attenuated E strain of Rickettsia prowazekii and subjects who had received Cox type killed R. prowazekii vaccine, in descending order of magnitude of titer which correlated incompletely with the protection afforded against typhus fever. However, it had been known for a good many years from the work of Giroud and others that patients convalescent from typhus fevers (and other rickettsial infections) reacted to the intradermal inoculation of killed organisms with a skin lesion which bore some similarity to the delayed type hypersensitivity to tuberculin in gross physical features and time course of development. Its use in the pre-CMI days was primarily as a tool for epidemiological studies. Emerging concepts of CMI suggested to us that this reaction might be of greater basic immunological significance than merely a convenient epidemiological tool. We confirmed in human subjects the general phenomenon, differentiated the non-specific endotoxin component from the immunologically specific component, defined its temporal kinetics and basic cellular composition. (Studies in man and animals clearly differentiated typhus endotoxic action, typhus mouse lethal toxic action and delayed type hypersensitivity one from the other.) In subjects inoculated with live attenuated E strain R.prowazekii, we demonstrated that skin tests became positive 7-10 days after inoculation, roughly about the same time that antibodies were first detectable in the blood. These observations, in the light of the rapidly evolving knowledge about CMI in general at that time, suggested to us that

cell mediated immune mechanisms might be important in immunity to typhus infections. It was against this background (1960's level) that the immunological component of the present research contract was begun.

2. Work under Current Contract.

a. Studies in Guinea Pigs.

Studies on the occurrence of cell mediated immunity and the relative importance of antibody-mediated (B lymphocyte) and cell mediated (T lymphocyte) immune responses in the control of typhus infections were initiated in the guinea pig-R. mooseri (Wilmington strain) model, conducted as two major sub-projects.

(1) The first project used the classical intraperitoneal route of infection and challenge with fever and scrotal reaction as indicators of infection or protection in male Hartley strain guinea pigs (≥750g). [This model required relatively large doses (10^3 PFU) of rickettsiae to induce a predictable and reproducible clinical response - 8-9 days of fever (rectal temp >39.5 C beginning 8 days after ip inoculation in naive or non-immune animals.]

Immune responses were measured in three treatment groups of guinea pigs: (1) animals vaccinated sc with 5×10^8 formalin-killed whole R. mooseri bodies; (2) animals vaccinated in the footpads with 10^9 formalin-killed whole R. mooseri bodies in complete Freund's adjuvant; and (3) animals inoculated ip with 10^3 viable R. mooseri. Immune responses were measured as follows. (1) Humoral (B lymphocyte mediated) response: the complement fixation and microagglutination tests for serum antibody. (2) Cell-mediated immune response correlates: the in vivo skin tests with 5 µg formalin-killed R. mooseri or soluble antigen for delayed type hypersensitivity (DTH) and the in vitro macrophage migration inhibition (MIF) test. (3) Protective immunity was measured by the suppression of fever and scrotal reaction following an ip R. mooseri challenge.

The results of this study, summarized in simplified form in the table below, indicated that guinea pigs developed both humoral (B cell mediated) and cell mediated immune responses to R.mooseri which varied with the nature of the induction stimulus. Thus, both infection and killed R. mooseri in adjuvant produced a strong antibody response and strong protection against challenge. The animals immunized with killed R. mooseri in adjuvant demonstrated both in vivo and in vitro correlates of CMI. However, the situation with guinea pigs infected with viable R. mooseri was complicated by the fact that these animals develop during infection with R. mooseri a factor in the serum which blocks the expression of both in vitro and in vivo correlates of cell mediated immunity, i.e. the skin test for DTH and the test

for MIF. However, the serum "blocking factor" did not prevent the expression of protective immunity. Significant is the fact that a sc dose of formalin-killed R. mooseri, of the order of the best R. prowazekii vaccines (previous contract), only induced a transient low antibody response and failed to induce detectable correlates of CMI or to provide protection against challenge.

Simplified Summary of Some Immunological Responses of Guinea Pigs to Different R. mooseri Preparations.

Treatment of Guinea Pigs	Antibody	DTH	MIF	Blocking Factor	"Protective" Immunity
Killed R. mooseri, no adjuvant	+ (low, transient)	0	0	0	0
Killed R. mooseri, complete adjuvant	+ (high, persistent)	+	+	0	+
Viable R. mooseri, infection	+ (high, persistent)	0	+	+	+

(2) The second series of guinea pig studies used a more natural (peripheral, intradermal) route of infection with R. mooseri and defined immunity in terms of control of rickettsial replication in, or clearance from, various tissues and organs in the infected animal, determined quantitatively by plaque counts. The use of inbred guinea pigs (Strain 2) permitted both antibody and cell transfer studies for determination of their relative role in the control of rickettsial replication in tissues.

Following intradermal inoculation into non-immune guinea pigs, R. mooseri underwent a most extraordinarily interesting sequence of cycles of rickettsial replication, control and clearance, first locally and then systemically:

At the local site of inoculation in the skin, the rickettsiae multiplied for the first 2-4 days, accompanied by the formation of a localized indurated lesion, after which their number declined rapidly to undetectable levels by 7 days. Local control was evident prior to the development of detectable serum antibodies. High titers of organisms appeared in the draining lymph nodes as the number of organisms at the inoculation site were declining. A rapid rise in antibody titers began just

before organisms were detectable, almost simultaneously, in blood, spleen and kidney. Rickettsemia was transient. The infection in the spleen underwent a separate cycle of rapid replication of organisms, control and clearance well after the similar cycle was complete in the skin at the site of inoculation and after serum antibodies had reached high titers.

Thus, some kind of immune mechanism is induced rapidly at the site of inoculation which controls the local replication of rickettsiae before antibodies are detectable in the blood. Subsequently, infection established in a distant organ (e.g., spleen) undergoes a similar cycle of replication, control and clearance despite the presence of rising serum antibody titers. These findings suggested that some immune mechanism other than serum antibody plays a dominant role in the control of rickettsial growth in tissue cells. (As in the previous project described above, skin tests for delayed hypersensitivity were consistently negative in these guinea pigs.)

The contribution the control of rickettsial replication in tissues of convalescent serum antibodies and immune spleen cells by following the fate of rickettsiae in inoculated skin of convalescent guinea pigs and of non-immune guinea pigs which had received large amounts of murine typhus convalescent serum or spleen cells from syngeneic convalescent spleen cells.

In guinea pigs convalescent from R. mooseri infection inoculated rickettsiae did not multiply but instead were rapidly cleared from the inoculation site. In naive guinea pigs which had received large quantities (up to nearly 50% of the estimated normal blood volume) of high-titered convalescent serum the inoculated organisms underwent an infection cycle of growth, control and clearance indistinguishable from that in control non-immune animals, clearly establishing the inability of antibody alone to control rickettsial replication in tissues. (The systemic spread of infection was not prevented, although there appeared to be some modification of spread, possibly through immune opsonization of free rickettsiae and their subsequent clearance by phagocytic cells - see below.) However, rickettsial replication was controlled at the site of inoculation in naive guinea pigs which had received spleen cells from convalescent, but not from normal, guinea pigs. This clearly demonstrated a dominant role for immune cells in the control of rickettsial growth in tissues. Because reagents for separating and identifying B and T lymphocytes were not readily available for the guinea pig but were becoming available for the mouse system, proof that the effector cells were indeed T lymphocytes was gained in the mouse system described below.

b. Studies in Inbred BALB/c Mice.

Studies of R. mooseri infection in BALB/c mice, summarized

below, confirmed the major immunobiological phenomena of the guinea pig system and extended them to (i) a clear identification of the effector lymphoid cells as T lymphocytes and (ii) a beginning exploration of the role of activated macrophages in the control of rickettsial infection.

(1) Immunobiology of R. mooseri infection following peripheral inoculation. R. mooseri infection initiated by subcutaneous injection was studied in BALB/c mice with the objective of developing a model for further studies of immune mechanisms. Local infection at the site of subcutaneous inoculation progressed through day 5 and was controlled by day 7. Systemic infection as determined by the presence of rickettsiae in the spleen was first detected on day 7 and progressed through day 14; however, rickettsiae persisted in this organ in reduced numbers through at least day 28. Control of the local infection at the site of subcutaneous injection occurred at about the time that humoral antibodies and hypersensitivity reactions to subcutaneously injected rickettsial antigens became demonstrable and was paralleled by a capacity to resist homologous subcutaneous challenge at a site distant from that of the primary infection. Systemic infection progressed in spite of this acquired immune capacity and was controlled in the spleen in parallel with the development of enhanced macrophage microbicidal capacity against Listeria monocytogenes in the liver. The results showed that an acquired immunity is capable of restricting rickettsial growth at subcutaneous sites at a time when rickettsiae are increasing in titer in deep organs.

(2) The mouse spleen model. A modification of the above mouse model was developed to explore specifically the individual contributions of immune (convalescent) serum, immune B and T lymphocytes and activated macrophages. The intravenous inoculation of a standardized dose of R. mooseri into naive BALB/c mice produced an infection in the spleen with rickettsial growth, co trol and clearance occurring in a reproducible and predictable r tern. Alterations in this pattern by adoptively transferred mune lymphoid cells, passively transferred immune serum or nonspecifically activated macrophages were readily detected and measured. The following main findings emerged from this powerful model.

(a) Passively transferred immune serum did not control established splenic R. mooseri infection.

(b) Adoptively transferred immune T lymphocytes, but not B lymphocytes, clearly controlled established splenic R. mooseri infection. An effective T lymphocyte possessed the capacity to divide.

(c) Neither activation nor ablation of macrophages altered the pattern and quantitative aspects of the growth and

subsequent control of splenic R. mooseri infection. Thus, splenic macrophages, activated by C. parvum or BCG (Mycobacterium tuberculosis) to express enhanced microbicidal action as measured by capacity to control Listeria monocytogenes infection, failed to control or reduce splenic R. mooseri infection. Conversely, the reduction or ablation of macrophages failed to interfere with the normal control of splenic R. mooseri infection.

c. Cellular Effector Mechanisms.

As the evidence was accumulating from the animal studies above to suggest that cell mediated mechanisms are dominant in the control of rickettsial infection at the intracellular level in tissues, studies were directed towards searching for effector mechanisms and included (1) soluble products of antigen-stimulated immune leukocytes (interferons, lymphokines, monokines, cytokines) which might influence growth of rickettsiae within host cells and (2) rickettsia-antibody-macrophage interactions. These studies were carried out in vitro with Rickettsia prowazekii and human antibodies and cells.

(1) Soluble factors with antirickettsial activity from typhus antigen stimulated human blood leukocytes (interferon gamma). Consideration of the architecture of the typical microvascular lesion in typhus suggested a possible role for diffusible soluble factors derived from the mononuclear cells of the lesion, presumably somehow involved in the expression of the cell mediated immunity, which could induce an antirickettsial action in the associated infected endothelial cells.

The supernatant fluids from R. prowazekii antigen stimulated blood leukocytes from human subjects who had previously experienced clinically overt typhus fever (R. prowazekii or R. mooseri infection), which were without any direct antirickettsial action on cell-free suspensions of extracellular rickettsiae, were found to cause progressive loss of stainable rickettsiae and evidence of host cell damage when added to R. prowazekii-infected human fibroblasts, peripheral blood-derived macrophages and umbilical cord endothelial cells but not to similarly infected chick embryo cells. Quantitative methods were devised to measure and characterize separately (a) the intracellular antirickettsial action and (b) host cell cytolytic action.

Some important findings in the extensive studies which were performed to characterize these two phenomena as expressed in cultures of R. prowazekii-infected human diploid fibroblasts included the following. Both actions were demonstrable in infected cultures of a wide variety of diploid and transformed human cells but not in cells of simian or murine origin. Both types of action could be induced by supernatant fluids from nonimmune leukocytes upon stimulation by phytohemagglutinin and by large doses of formalin-killed typhus rickettsial bodies (?

lipopolysaccharide). Disappearance of stainable rickettsiae from infected cells was paralleled by a loss of infectious units. Expression of the intracellular antirickettsial action, but not the cytolytic action, required prior synthesis of newly transcribed host cell proteins. The cytolytic action was selective for rickettsia-infected cells in mixed cultures. Conversion of the fibroblasts from cytolysis-resistant to cytolysis-susceptible was induced by the entry of only a very few viable rickettsiae (1-3) into the host cell cytoplasm, even when rickettsial growth and protein synthesis was inhibited with chloramphenicol.

The supernatant fluids from antigen-stimulated immune leukocytes are known to contain a wide variety of lymphokines and monokines which have diverse actions. The findings described above initially suggested the possibility of two different factors: (a) an interferon-like factor inducing antirickettsial action and (b) a cytolytic factor selective for infected cells. Neutralization studies were performed with polyclonal antibodies against IFN-α, IFN-β, IFN-γ and lymphotoxin, subsequently confirmed with monoclonal antibodies, revealed that anti-HuIFN-γ antiserum, but none of the others, neutralized **both** the intracellular antirickettsial action and the cytolytic action on infected cells induced by supernatant fluids from antigen-stimulated immune leukocytes and from PHA-stimulated nonimmune leukocytes. Moreover, highly purified natural HuIFN-γ, but not IFN-α or IFN-β, reproduced both types of action, as did recombinant IFN-γ.

These findings suggested that IFN-γ may be one of the effector mechanisms of cell mediated immunity in typhus infections. It induces endothelial and other cells to kill intracellular rickettsiae and it activates macrophages to kill rickettsiae (now identified by others as classical macrophage activating factor - MAF).

(2) <u>In vitro</u> Observations on Rickettsia-Antibody-Macrophage Interactions. Although the animal studies described above minimized the importance of antibodies and macrophages in the control of **intracellular** rickettsiae in established infections in tissues and previous studies had shown that antibodies present in human typhus convalescent serum, with or without added complement failed to kill typhus rickettsiae and that non-activated macrophages in the absence of antibodies did not kill typhus rickettsiae, neither antibodies nor macrophages can be dismissed out of hand as playing no role in the control of typhus infections. Both <u>R. prowazekii</u> and <u>R. mooseri</u>, when opsonized by antibodies present in human convalescent serum, are avidly phagocytized by non-activated macrophages in culture and are rapidly destroyed, thus suggesting a role in the clearance of **extracellular** rickettsiae in the classical sense. (Macrophages obviously probably have other roles in the immune response, such as antigen presentation and the elaboration of various factors

which participate in the modulation of some immune processes.) Indeed, the guinea pig studies documented above suggested that immune serum passively transferred to naive guinea pigs altered through blood-borne distribution of R. mooseri to distant organs. Accordingly, an in vitro study was undertaken to investigate rickettsia-antibody-macrophage interactions more fully, relying heavily upon an ultrastructural approach.

A carefully standardized, reproducible in vitro system was developed for this study, employing human peripheral blood monocyte-derived macrophages in defined stages of evolution into macrophages, a special R. prowazekii seed enriched for viable rickettsiae so as to minimize the distortion in ultrastructural studies caused by dead rickettsiae, and the serum of a subject known to be solidly immune to typhus. In ultrastructural studies of lysosomal fusion, acid phosphatase and the thorium dioxide particles incorporated into secondary lysosomes were used as markers. Quantitative time-course experiments formed the major basis for this study. Some of the salient findings of this study are outlined below.

When viable R. prowazekii were taken up by macrophages in the presence of nonimmune serum, a substantial proportion of the macrophages were killed - a cytotoxic effect of the rickettsiae. In the surviving macrophages, the rickettsiae were initially found in membrane bound phagosomes but within minutes a defect was induced in the phagosomal membrane through which the rickettsiae escaped into the macrophage cytoplasm where they replicated and eventually destroyed the macrophage. There was no evidence of fusion of lysosomes with the rickettsia-containing phagosomes, suggesting the possibility of rickettsial inhibition of lysosomal fusion.

When viable R. prowazekii pretreated with typhus immune serum were taken up by macrophages, the rickettsiae were retained in the phagosome and there was rapid fusion of lysosomes with the phagosomes with lysosomal content markers surrounding the rickettsiae in the phagolysosome. Within minutes, the rickettsiae within the phagolysosomes showed structural changes, especially a condensation consistent with loss of cytoplasmic membrane integrity, and thereafter underwent progressive morphological degradation.

Macrophages derived from the blood of patients with chronic granulomatous disease, deficient in activated oxygen intermediate mediated microbicidal mechanisms, destroyed immune serum-treated rickettsiae in a manner indistinguishable from that observed with macrophages from normal human subjects.

Suspensions of purified viable cell-free R. prowazekii showed no detectable catalase activity and failed to generate detectable hydrogen peroxide when actively oxidizing glutamate.

The findings of this study, together with those of studies under the previous contract and those described in the section above, support the following conclusions.

(a) Virulent R. prowazekii escapes destruction by non-activated human peripheral blood monocyte-derived macrophages in vitro by suppressing lysosomal fusion and rapidly escaping from the phagosome into the cytoplasm through a rickettsia-induced defect in the phagosomal membrane.

(b) Virulent R. prowazekii, pretreated with human typhus convalescent serum, is avidly phagocytized by non-activated human peripheral blood monocyte-derived macrophages, is retained within the phagosome with which lysosomes rapidly fuse, and is rapidly destroyed within the phagolysosome. Since immune serum has no direct rickettsiacidal action, it is reasonable to suggest that it acts intraphagosomally by "neutralizing" rickettsial inhibition of lysosomal fusion and of the formation of the phagosomal defect.

(c) Killing and degradation of intraphagosomal antibody-treated rickettsiae in non-activated human peripheral blood monocyte-derived macrophages appears to be mediated through lysosomal components independent of the microbicidal action of activated oxygen intermediates.

(d) Human peripheral blood monocyte-derived macrophages activated by HuIFN-γ kill ingested virulent R. prowazekii in the absence of typhus convalescent serum, suggesting a different mode of rickettsiacidal action.

(3) Brief Resume and Implications for Immunological Requirements for Typhus Vaccines. Cumulative experience with typhus infections in animals and Man as well as in vitro experiments with rickettsiae, antibodies and cells clearly defines the following immunobiological phenomena.

(a) During the course of typhus infection, rickettsia-specific antibodies are formed, macrophages are activated and rickettsia-specific T lymphocytes are produced. Specific antirickettsial antibodies that develop during infection and macrophages appear to act on **extracellular** rickettsiae, whereas T lymphocyte mediated mechanisms appear to act upon **intracellular** rickettsiae. The information accumulated to date suggests the following roles for these components of the immune response.

The immune mechanisms directed at clearance and killing of extracellular rickettsiae appear to be of limited effectiveness and, of themselves, only modify, but do not prevent, the systemic dissemination of organisms, possibly due to the fact that even in the blood many rickettsiae may be in inaccessible intracellular

sites.

(i) Although antibodies and complement do not kill rickettsiae and do not prevent them from infecting cells that are not professional phagocytes, they do opsonize the rickettsiae to which they have access and prepare them for retention and killing within macrophage phagolysosomes. Passive transfer of very large quantities of immune serum only modified systemic dissemination of infection in guinea pigs and failed to control rickettsial growth in tissues in both guinea pigs and mice.

(ii) Macrophages, non-activated and in the absence of appropriate antibodies, do not kill virulent typhus rickettsiae (suppression of lysosomal fusion and escape from phagosome) but instead support their growth and are killed by them. However, in conjunction with either B lymphocyte mediated mechanisms (antibody-mediated opsonization and retention in rickettsiacidal phagolysosome) or T lymphocyte mediated mechanisms (e.g., "activation" by IFN-γ), macrophages can kill rickettsiae to which they have access, which may be limited. Non-specifically activation of macrophages for enhanced microbicidal action failed to prevent intravenously administered rickettsiae from establishing infection and from growing in the spleen of mice.

Only T lymphocyte mediated immune mechanisms effectively controlled the growth of rickettsiae and killed them within their natural host cells in the infected animal. Unfortunately, this contract expired before the mechanisms by which cell mediated immunity controlled rickettsial infections could be fully explored. However, it was found that rickettsial antigen stimulation of immune cells from the blood of persons convalescent from typhus infections caused the elaboration of a soluble product, identified as IFN-γ, that caused the death of R. prowazekii growing within human umbilical endothelial cells, macrophages and fibroblasts and caused the lysis of infected, but not uninfected, cells. Thus, at least one mechanism for the expression of cell mediated immunity in typhus has been identified.

(b) Typhus vaccines which do not induce the development of specific cell mediated immunity have limited protective potency. Guinea pigs vaccinated with 5×10^8 formalin-killed R. mooseri, a larger rickettsial content than most typhus vaccines of the Cox type, produced modest levels of humoral antibodies, little or nor evidence of CMI (MIF production) and failed to resist challenge. Similar vaccines have been incompletely protective in Man against typhus and spotted fever. When administered to guinea pigs with Freund's complete adjuvant, however, such vaccines induced strong CMI and protected against challenge. The living E strain R. prowazekii vaccine induces in human beings a modest antibody response, CMI as measured by skin tests for delayed type hypersensitivity, and strong protection

requirement for a truly protective typhus vaccine is the capacity to elicit an effective cell mediated immunity against appropriate typhus components.

D. Studies on Surface Components of Rickettsia prowazekii. In the course of the above described studies on the immunobiology of typhus, it became apparent that pr cise knowledge of the surface components of R. prowazekii which might be immunogens of importance in inducing protective immunity would become essential to the study and that for the first time satisfactory methods had been developed for the separation and characterization of proteins - e.g., slab polyacrylamide gel electrophoresis (PAGE), immune precipitation and monoclonal antibodies. Accordingly, this matter was pursued in parallel with the ongoing immunobiological studies.

The cell envelope as the location of immunologically important components in typhus rickettsiae had its origin in these laboratories nearly two decades earlier when it was shown that the cell envelope of R. mooseri contained components which induced the production of toxin neutralizing antibodies in mice, CF antibodies and resistance to challenge in guinea pigs, lipopolysaccharide (endotoxin) and antigens which elicited delayed type hypersensitivity in skin tests in human subjects convalescent from laboratory proven murine typhus fever.

The studies of this report were done with R. prowazekii grown in cell cultures whose protein synthesis was inhibited with cycloheximide or emetine, permitting the specific labeling of rickettsial proteins with ^{3}H-leucine which could then be separated by PAGE and visualized on fluorograms without the need of complex purification. Surface location was established by the surface labeling of purified rickettsiae with ^{125}I followed by PAGE.

Immune precipitations of radiolabeled R. prowazekii (Breinl strain) proteins with typhus immune serum identified 3 major polypeptide antigens (apparent M_r = 138 kd, 32 kd and 31 kd).

Five R. prowazekii strains with different passage histories and sources of isolation had identical polypeptide antigen profiles by PAGE.

PAGE analysis of the attenuated E strain of R. prowazekii revealed that the 31 kd polypeptide from either ^{3}H-leucine- or ^{125}I-labeled rickettsiae had a slightly lower apparent M^r when compared to the virulent strains. Partial proteolytic digests of the virulent Breinl- and attenuated E-31 kd polypeptides (^{125}I-labeled) indicated that the structural difference lies in a region of these polypeptides exposed on the rickettsial surface. Solubilization of the 31 kd polypeptide with detergent solutions containing guanidine HCl or β-mercaptoethanol indicated that the

31 kd polypeptide is tightly bound to the rickettsial outer membrane, similar to the matrix proteins or porins of other gram negative bacteria.

Purified, infectious R. prowazekii (Breinl) was used to immunize BALB/c mice for the production of monoclonal antibodies. The antigen specificity of most monoclones was against the 138 kd surface polypeptide. Another antigen, of unknown biochemical composition, was recognized by a different monoclone and was designated "diffuse" antigen because of it's diffuse distribution in the cytoplasm of infected cells as observed by fluorescent antibody staining.

The reactivity of 7 monoclones (6 against the 138 kd polypeptide and 1 against the "diffuse" antigen) were tested in several classical serological tests, using the rickettsial antigen conventionally employed in each type of test. Monoclonal antibodies against the 138 kd polypeptide reacted in the homologous microagglutination, complement fixation (both "soluble" and particulate antigens) and the indirect immunofluorescence tests. The reactions were specific for the respective R. prowazekii antigens only with 6 monoclones and were also cross-reactive with R. mooseri antigens with 2 monoclones. Thus, there seem to be both R. prowazekii-specific and typhus group specific epitopes on the R. prowazekii 138 kd polypeptide.

The monoclonal antibody against the "diffuse" antigen reacted with both R. prowazekii and R. mooseri in the microagglutination, complement fixation ("soluble" antigen) and the indirect immunofluorescence tests.

The same small battery of 7 monoclonal antibodies was used to investigate the possible role of the 138 kd polypeptide and the "diffuse" antigen in a series of rickettsial biological activities: rickettsial uptake, hemolytic activity, complement assisted rickettsiacidal action, lethal toxic action for mice, opsonization and destruction within human macrophages in culture.

The monoclonal antibody against the "diffuse" antigen had minimal to negligible effects on all the activities studied.

The monoclonal antibodies against the 138 kd polypeptide showed varying patterns of reactivity. Thus, 5 of the 6 uniformly neutralized mouse lethal toxic activity and exhibited opsonization and enhanced destruction within macrophages. One monoclone exhibited none of these activities. The results with effects on other biological activities were more difficult to interpret. The same 5 monoclones either failed to influence rickettsial uptake or enhanced it. Some increased plaque formation, had no effect or slightly reduced it. The other monoclone strongly inhibited both uptake and plaque formation. The inhibitory action on rickettsial hemolysis ranged from slight

to modest for all 6. There was a suggestion that certain monoclones, when supplemented with complement, may exhibit some degree of rickettsiacidal action.

Although further work is necessary to clarify the actions of these monoclonal antibodies against the 138 kd polypeptide on various biological properties of typhus rickettsiae, **it is clear that the 138 kd polypeptide is a major surface component which is involved in rickettsial interactions with host cells and macrophages.**

Critique. There is a major conceptual flaw in the approach taken in this project. Historically, it was initiated before the dominant role of cell mediated mechanisms in typhus immunity was established. The 138 kd surface polypeptide was selected for study because human convalescent typhus serum contains antibodies against it and because clones producing antibodies against it dominated in the mouse in the procedure for making monoclonal antibody. Attention was focussed on the classical approach of finding an immunogen which would induce the production of antibodies which interfere with some putative essential rickettsial function. This may be irrelevant. What is now known to be needed is an immunogen which will induce the production of appropriate T lymphocytes bearing receptors for that immunogen which, when stimulated by invading rickettsiae bearing the appropriate antigenic determinants, will initiate the production of the soluble factors and cytotoxic cells which will kill intracellular rickettsiae and destroy infected cells! It remains to be determined if this 138 kd rickettsial surface polypeptide, when properly presented as an immunogen, will induce protective cell mediated immunity. It will indeed be fortuitous if it does.

E. DNA:DNA Hybridization Studies and Elementary Genetics.

1. **DNA:DNA Hybridization Studies.** Prompted by the difficult problem of classifying our spotted fever group rickettsial isolates from Pakistan (isolated under a previous contract), we began to explore the use of DNA:DNA hybridization methods to establish broad relationships among rickettsiae of the spotted fever and typhus groups. Two methods were worked out: (1) an optical method which not only yielded information on the degree of hybridization but also permitted the calculation of base ratios and genome size and (2) a radioisotope method which required much smaller quantities of DNA. Radiolabeling of the DNA was performed by the nick-translation method. Production of sufficient quantities of rickettsiae of the typhus group for these studies by growth in the yolk sac of embryonated eggs was no special problem. However, the production of adequate quantities of the species and isolates of the spotted fever group, whose yields in embryonated eggs were 1-2 logs lower than typhus group rickettsiae, required an enormous, sustained effort over a period of 2-3 years. This study has yielded the following information.

a. Data on genome size and base composition was obtained for many of the rickettsiae of the typhus and spotted fever groups for which published data were not available. The new data followed the patterns previously observed for each group and reinforced the hypothesis that members of each of these two groups, established on the basis of phenotypic properties, possessed genomes with overall general similarities characteristic of the group. The genome of Coxiella burnetii differed substantially from that of the typhus and spotted fever groups.

b. In the **typhus group**, there was only about 72-75% DNA homology between R. prowazekii and R. mooseri. This difference might be expected to have occurred by divergence on an evolutionary time scale. It is inconsistent with the hypothesis still persisting in some corners that R. mooseri can transform into R. prowazekii frequently and rapidly after a few passages in lice. The improbability of regular simple mutations from R. mooseri to R. prowazekii has considerable practical importance with respect to control measures for louse-borne typhus because it tends to exclude a major distraction for public health officials concerned with the control of louse-borne epidemic typhus - viz., that the R. mooseri endemic in the rodent populations is a constant potential source of R. prowazekii in a lousy population - and permits attention and resources to be directed at the real source of the problem. (This does not exclude the possibility that under certain circumstances R. mooseri might be transmitted as R. mooseri by human body lice.)

c. On the basis of the homology studies, the **spotted fever group** clearly fell into 3 major subgroups, comparable in general to those identified by various investigators through serological affinities and PAGE analysis of major proteins, but the species within the subgroups could not be differentiated. Thus, DNA:DNA homology between whole genomic DNAs is useful for the study of broad relationships within this group.

(1) Rickettsia rickettsii-R. conorii complex. Two of our Pakistan tick isolates, an Indian tick typhus strain and an Israeli tick typhus strain also fell into this subgroup.

(2) Rickettsia sibirica complex. Two of our Pakistan tick isolates and the Thai tick typhus strain fell into this subgroup.

(3) Rickettsia akari-Rickettsia australis complex.

d. The typhus and spotted fever groups showed about 40-50% DNA homology. Rickettsia canada, commonly regarded as a peculiar member of the typhus group, stands in an apparently anomalous position, showing only about the same degree of homology with both typhus and spotted fever groups as the two groups exhibit

between each other. The taxonomic and evolutionary significance of this finding remains to be elucidated.

e. DNA homology studies with Rochalimaea quintana and Baker's vole agent, now known as R. vinsoni are described below with the other studies that formed the basis for accepting the latter as a separate species instead of a strain of the former as had been proposed by others.

2. **Elementary Genetics.** Except for the much earlier work of Weiss on the selection of antimicrobial resistant strains of R. prowazekii by serial passage in eggs, essentially nothing was known of classical type rickettsial genetics. Spurred by the recognition of some potential problems with antibiotics in typhus fever in the field, we performed some preliminary studies on some genetic properties of Rickettsia prowazekii, making use of the great improvements in quantitative rickettsial methodology and resistance to antirickettsial antibiotics as markers.

a. **Mutant Frequency of Antibiotic Resistance.** The classical experiment of Demerec of antibiotic resistant mutant frequencies in bacteria at increasing drug concentrations was adapted to the rickettsial system. The plaque reduction method (see below) was modified for use here and R. prowazekii (Breinl) was the organism. A practical lower limit of measuring antibiotic resistant mutant frequencies of about 10^{-5} was imposed by the technical problem of massive lysis of the CE cell monolayers by the large inocula necessary to provide a rickettsial population large enough to measure mutant frequencies at higher antibiotic concentrations. Nevertheless, up to this limit, the antibiotic concentration/mutant frequency patterns observed with R. prowazekii followed general patterns described for bacteria. The theoretical significance of this with respect to the possibility of the development of antibiotic resistant strains of R. prowazekii in nature under appropriate selective conditions, as we have demonstrated to be possible in infected body lice feeding upon a person under antibiotic therapy, is evident.

b. **Selection of Penicillin Resistant R. prowazekii (Breinl).** By serial passage of R. prowazekii (Breinl) in CE cell cultures containing increasing concentrations of penicillin G (benzyl penicillin), it was possible to select and isolate a clone that was 50-100 times more resistant to penicillin G than was the parent strain.

c. **Search for Plasmids in R. prowazekii (Breinl).** The DNA from R. prowazekii (Breinl) was examined by cesium chloride equilibrium density centrifugation and by agarose electrophoresis for the presence of plasmids. None was found.

d. **Attempts at Transformation.** Several attempts were made to demonstrate transformation in R. prowazekii, using the

penicillin resistant virulent Breinl strain described above and the erythromycin resistant attenuated E strain developed by Weiss some years previously. Detection of dually resistant transformants relied upon the detection of plaques in plates containing both antibiotics in the overlay medium. Although the use of the antibiotic resistance markers was a convenient and simple means of detecting the phenomenon of transformation, the use of virulent and attenuated strains would have facilitated the extension of the system, if successful, to the study of the genetic basis of virulence. Two methods for examining for transformation were employed: (1) the classical method employed for bacteria of exposing **cell-free** organisms in suspension under the then defined optimal conditions to DNA from organisms bearing the desired genetic marker and then examining for organisms bearing the marker present in the DNA preparation and (2) a method devised by us for detecting **intracellular** transformation. The latter depended upon dually infecting host cells with erythromycin-resistant and penicillin resistant strains, causing intracellular release of the DNA from the erythromycin resistant strain by the formation of unstable spheroplasts under the influence of penicillin in the medium (see below), and the subsequent search for dually resistant organisms. This was a technically demanding method depending heavily upon the predictability of infection, intracellular growth and kinetics of spheroplast formation made possible by the methodology developed in these laboratories. It was postulated that among organisms growing intracellularly some might be in a special receptive state for transformation. Unfortunately, in a limited number of trials no evidence was obtained for successful transformation, either extracellularly or intracellularly. Possibly more recent information about inducing the receptive state might permit the development of better test systems.

F. Chemotherapy of Rickettsial Infections. The chemotherapy of rickettsial diseases has overlapping clinical and epidemiological objectives, depending upon the agent, characteristics of the infection and the role that Man plays in the transmission cycle. Reduced to the simplest form, these objectives would be met by ant rickettsial drugs which would cause (1) rapid termination of the clinical disease (acute, chronic or late) and (2) eradication of the organisms. None of the currently accepted drugs (chloramphenicol and the tetracyclines), thought to be primarily rickettsiastatic, meets all of the criteria. In the case of louse-borne epidemic typhus (R. prowazekii infection), they rapidly and dramatically terminate the clinical disease and reduce morbidity and mortality; they probably rapidly terminate rickettsemia, thereby interrupting the louse-man transmission cycle when louse control is not complete or even possible; but they probably do not eradicate the organisms and prevent the latent infection which later gives rise to recrudescent typhus (Brill-Zinsser disease) with its clinical and epidemiological consequences. Drugs with significant rickettsiacidal action

might be effective in eradicating the organisms. Our interest in these problems of chemotherapy of rickettsial diseases began with the early Malaya studies on chemotherapy and chemoprophylaxis of scrub typhus and have continued as advances in antimicrobial agents in general and in rickettsial methodology have occurred and field opportunities have presented themselves.

a. **In Vitro Search for Antirickettsial Drugs.**

Methodological advances in reproducible, predictable and quantitative in vitro rickettsial methods (described above) permitted adaptations for the observation and measurement of the effects of antimicrobial agents on rickettsiae. Two basic methods were developed for examining the action of antimicrobial agents on intracellular rickettsiae.

(1) The **Slide Chamber Culture Direct Count Method** permitted the measurement of the effect of introduced antimicrobial agents on the intracellular growth of rickettsiae, on morphological alterations that might be induced and on the resumption of growth, if any, on removal of the drug-containing medium.

(2) Two variations of the plaque count method were developed. (a) The **Plaque Reduction Method**, in which the number of plaques that developed in a monolayer inoculated with a standard number of rickettsial PFU under agarose overlays containing graded antibiotic concentrations were compared with the number that developed in the absence of antibiotic, provided information on the **rickettsiastatic** action of an antibiotic comparable to the minimal inhibitory concentration (MIC) determined for extracellular bacteria. (b) The **Delayed Overlay Method**, in which inoculated monolayers were first covered with antibiotic in fluid medium for the desired period of time and then after washing were covered with antibiotic-free agarose overlay medium to allow development of plaques by the surviving organisms, is a quantitative method for determining the **rickettsiacidal** action of an antibiotic and can be run either as a rate or as a concentration gradient test.

Using both above methods for measuring rickettsiastatic concentrations, the MIC(µg/ml) of the following antibiotics was determined for R. prowazekii (Breinl), listed in order from lowest to highest MIC: rifampin (0.008 µg/ml); erythromycin (0.06 µg/ml); doxycycline, minocycline, tetracycline (all ~ 0.1 µg/ml); chloramphenicol (1.0 µg/ml); penicillin G (20-50 µg/ml).

Using the delayed overlay method for rate of rickettsiacidal action at a concentration ≥ 10x the MIC (still clinically attainable), it was found that chloramphenicol, doxycycline, erythromycin and rifampin exhibited a slow but definite rickettsiacidal action which, however, appeared to be too slow for eradication of

typhus rickettsiae under the usual therapeutic regimens.

Although the MIC for penicillin G was somewhat above that which was practical clinically, this antibiotic was given further attention because light microscope examination of the slide chamber tests, confirmed by transmission electron microscopy of ultrathin sections, revealed that the rickettsiae transformed into unstable intracellular spheroplasts in the presence of penicillin G. Delayed overlay rickettsiacidal tests revealed a **rapid rickettsiacidal** action. Subsequently, a large series of semisynthetic penicillins and cephalosporins was screened for action on intracellular R. prowazekii at clinically practical concentrations but none was found to be superior to penicillin G.

Screening tests by the plaque reduction method at the single concentration of doxycycline of 0.1 µg/ml revealed that all of the established species of the spotted fever group of rickettsiae were inhibited. Slide chamber studies with the same panel of spotted fever group species showed that all underwent spheroplast transformation in the presence of 100 µg/ml penicillin G.

b. **Clinical Studies on the Treatment of Louse-borne Typhus.** The epidemic of louse-borne typhus in Burundi, which provided the opportunity for the successful controlled, WHO sponsored and Army supported, field trial of the attenuated living E strain typhus vaccine reported under the previous contract, also provided the opportunity for a systematic study of the chemotherapy of typhus fever which extended well into this contract.

The chemotherapy study was initiated in mid-1969 coincident with the beginning of the controlled E strain vaccine trial and continued well into the early years of this contract. The main base of operation for the vaccine trial and this study was the Catholic Mission Dispensary at Katara (Gatera), Ngozi Province in Burundi which was operated by the White Sisters of A rica. Sr. Jessy Goosse was in charge of the typhus patients admitted for treatment and worked as an integral part of the field team. Her remarkable skill at identifying typhus patients among the many other febrile illnesses was amply confirmed later by our laboratory in Baltimore. Additional small series of patients were treated according to protocol at other missions by Sisters who were trained at Katara with indistinguishable results.

The basic design of the study was as follows. From the large number of febrile patients who presented each morning at the dispensary, those who appeared to be suffering from moderately severe to most severe typhus fever on the basis of clinical diagnosis were entered into the study. Serum specimens were obtained upon admission, upon departure and later, if possible. Serological confirmation of diagnosis, usually possible by rise in antibody titer between admission and discharge, was made at a later date in Baltimore. A standardized form, developed for this

purpose, was used to record daily temperature and a variety of relevant symptoms and physical findings. Some patients had other infections in addition to typhus, notably dysentery and malaria (confirmed by blood smear). These were treated specifically as indicated coincident with antityphus therapy. The most critical differential diagnostic problem on admission was the comatose patient who could be suffering from cerebral malaria, typhus or both. Emergency priority in these patients was given to the detection of malarial parasites in blood smears and, if positive, immediate administration of parenteral chloroquine (This antedated the appearance of chloroquine resistance in the area) followed promptly by antityphus therapy. Some patients with typhus were pregnant. Follow-up as possible was made to detect abortions associated with typhus.

The original plan was to compare the efficacy of chloramphenicol and tetracycline HCl in the treatment of typhus. However, just prior to leaving Baltimore for Burundi, the tetracycline derivative, doxycycline, which was claimed to produce prolonged blood levels and require only one or two doses a day for the treatment of various infections. A supply of this new drug was obtained from Pfizer International for testing because an effective antityphus drug which would require only one dose a day would greatly facilitate the management of large numbers of typhus patients under extremely primitive conditions and a great shortage of medical personnel.

Only a few patients were treated on a rotational basis with chloramphenicol, tetracycline HCl and doxycycline before the great advantage of doxycycline became apparent and all efforts were directed towards defining the minimum dose requirements for doxycycline. The initial regimen was a single 100 dose daily until the patient had been afebrile for 48 hours. The number of days of treatment was gradually reduced until it was found that one single 300 mg oral dose of doxycycline would cure typhus fever as rapidly and without relapse as conventional therapy. Then it was found that **the dose of doxycycline could be reduced to a single 100 mg capsule in adults and a single 50 mg dose in children to yield the same prompt therapeutic control of typhus.**

Because typhus was in epidemic form in many parts of Burundi (as well as in Rwanda and some other areas) and posed a major burden to the limited medical facilities and resources, knowledge of the fact that typhus could be treated with a single dose of doxycycline was freely disseminated throughout the region and permission was given to Pfizer International to disseminate the information while our study was still in progress and before there had been opportunity to publish the findings. Very soon published reports on the phenomenon began to appear, only one or two acknowledging the original source of the information.

The work on doxycycline continued, with the objective of

documenting an adequate, significant number of laboratory confirmed cases to substantiate our claims. When this was done, a similar study was performed with minocycline, a related tetracycline derivative with comparable pharmacokinetics which also had just become available. The results were identical-viz., **a single 100 mg dose of minocycline would also cure louse-borne typhus.**

Some typhus patients who were carried into the dispensary for treatment did not wish to be admitted. These were treated as outpatients with a single dose of doxycycline after which they were carried back to their place of abode. Others were treated similarly in their homes, requiring only a single visit by a medically trained person. To the extent that follow-up was possible in such instances, it appeared that this form of therapy satisfactorily cured typhus in the otherwise healthy patient.

It is desirable to have alternative drugs available for the treatment of typhus in the patient who may not be able to take a tetracycline or chloramphenicol or in the event that a resistant strain is being transmitted (see above). The in vitro studies above disclosed two additional drugs with very high rickettsiastatic action: erythromycin and rifampin.

Although erythromycin already had proven to be unreliable in the treatment of Rocky Mountain spotted fever, the very low MIC for *R. prowazekii* suggested that it might be worthy of a trial. Unfortunately, it proved to be so unreliable when given orally in typhus that further testing was abandoned after the first 4 patients.

On the other hand, rifampin given orally in two 300 mg doses per day until the patient had been afebrile for 48 hours produced a therapeutic response in typhus which was indistinguishable from that produced by chloramphenicol or drugs of the tetracycline series. Thus, **although rifampin is not recommended for the routine treatment of typhus, it stands as an effective alternative in the event that one is required.**

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